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BIOPHYSICAL AND BIOCHEMICAL MECHANISMS IN SYNAPTIC
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R R LLINAS 28 SEP 87 AFOSR-TR-87-1496 AFOSR-85-0360

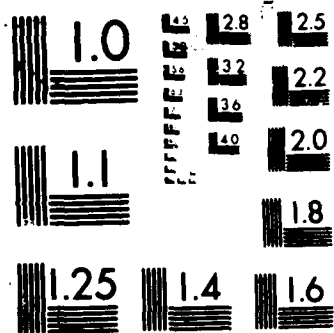
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<p>Four major goals were accomplished in the second year. Two related to synapsin I regulation of transmitter release and two related to the temperature dependence of the synaptic release process with a preliminary study of quantal release at the squid giant synapse. The results may be summarized as follows: (1) Demonstration that the dephosphorylated tail fragments of synapsin I do not regulate synaptic release which excludes the possibility that tail fragments themselves can prevent vesicular release. (2) Injection of head phospho synapsin I does not regulate synaptic release, demonstrating that the molecule itself does not interfere with vesicular fusion. These two experiments indicate that synapsin I works by affixing the synaptic vesicles to the cytoskeletal system. (3) Video-enhanced microscopy results demonstrated that</p> <p>(continued on reverse of page)</p>				
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axoplasmic mobility is not altered by either tail fragments or head phospho synapsin I, confirming the findings obtained by the electrophysiological study. The results demonstrate the mechanism by which synapsin I regulates transmitter release. (4a) A study of the temperature dependence of transmitter release demonstrated that temperature can be used to study the kinetics of synapsin I inhibition of transmitter release. (4b) Measurements of miniature potentials were finally accomplished routinely and can now be utilized as a technique to determine directly the effect of synapsin I on single vesicular fusion.

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AFOSR-85-0368 (Rodolfo R. Llinas)

Interim Scientific Report - 30 Sept 87Summary

Four major goals were accomplished in the summer of 1987. Two of these related to the mechanism by which synapsin I regulates transmitter release at chemical synapses. The other two had to do with measurement of the temperature dependence of the release process, a mechanism that must be understood to further clarify the role of synapsin I in synaptic release and a preliminary study of quantal release at the squid giant synapse. Relating to the synapsin I study the major finding was the demonstration that synapsin I regulates synaptic transmission *only if the molecule is intact*. Thus, injection of tail fragments of synapsin I into the terminal, while capable of binding vesicles, did not produce a significant change of the release process, indicating directly that synapsin I does not act by interfering with the vesicle plasmalemmal fusion.

In a second set of experiments synapsin I phosphorylated at the head was injected and demonstrated not to interfere with synaptic release. These two sets of experiments demonstrate, therefore, that the results described in last year's research (i.e. that synapsin I inhibits transmitter release) requires for this effect that both head and tail portions of synapsin I be dephosphorylated and the head and tail fragments of the molecule be attached to one another. These results give further support to the model initially presented (Llinas et al.) that synapsin I must regulate vesicular mobility by fixing the vesicular membrane via its tail while its head binds to the endoskeletal system (probably actin filaments) in order to form a 3-dimensional web at the release site. The above results were also confirmed by video-enhanced microscopy. In collaboration with Theresa McGuinness from Paul Greengard's

laboratory, we demonstrated that the results obtained by intracellular injection of the different forms of synapsin I described above had no effect on vesicle and organelle mobility in the extruded squid axoplasm, as described last year and confirmed this year. Dephosphorylate synapsin I totally blocked axoplasmic flow while injection of phosphorylated synapsin I had no effect on this mobility.

The third study consisted in determining the effects of temperature on synaptic release. This set of experiments was particularly significant as the temperature effect can give an important insight into the mechanisms regulating the release process. Studies were done which covered a temperature range from 1 to 23°C and the parameters measured included rate of rise, amplitude and latency of the postsynaptic response. The results indicate that the relation between temperature and synaptic latency and rate of rise may be described by a single exponential and the Q10 for the reaction could be as high as 4 from 5° to 15° C decade.

Finally a preliminary set of experiments was performed on the post-synaptic elements of the squid giant synapse which indicate the possibility of measurement of miniature synaptic potentials at this junction. This technique will be very effective in this study of synapsin I on transmitter release.

Statement of work

The first objective in the second year of research was to confirm our results of 1986, i.e. that dephosphosynapsin I blocks transmitter release and that the phosphorylated form of this protein does not. The project began in 1987 by repeating some of the experiments which basically confirmed the initial results and gave sufficient data to consider this portion of the project as having been accomplished.

The central question asked in 1987 related to the *mechanism by which dephosphosynapsin I regulates transmitter release*. Two major hypotheses were proposed and new experiments had to be designed to determine which of the two mechanisms was in fact at work. The first hypothesis assumes synapsin I had to be released from the surface of vesicles before they could fuse to the plasmalemmal membrane. This release had to be accomplished by calcium entry through the presynaptic calcium channels via the activation of calmodulin dependent kinase II known to phosphorylate the tail portion of the molecule.

The other possibility would be that if a sufficient number of synapsin I molecules could be phosphorylated the vesicles could then approach the plasmalemmal membrane and fuse with it despite the fact that synapsin I molecules were still on the surface of the vesicle.

Experiment. 1. In order to determine which of the two possibilities actually were at work, tail fragments from synapsin I (prepared in Dr. Greengard's laboratory) were injected into the presynaptic terminal, and following there marked with Texas red. This set of experiments demonstrated that the tail segments of synapsin I, while capable of adhering to vesicles in the dephospho form, do not interfere with the release process. An example of this type of experiment is illustrated in Fig. 1. The results of the experiment, being very clear, indicate that the tail fragment does not interfere with release.

Experiment 2. In the second set of experiments synapsin I molecules were phosphorylated at the head leaving the tail nonphosphorylated. A typical result is shown in Fig. 2 where synapsin I was injected following marking with Texas red which allowed a correlation between site of injection and the diffusion of protein into the presynaptic tail with the amount of synaptic release. The results illustrated in Fig. 2 demonstrate that synapsin I phosphorylated at the head does not block synaptic transmission. This indicates

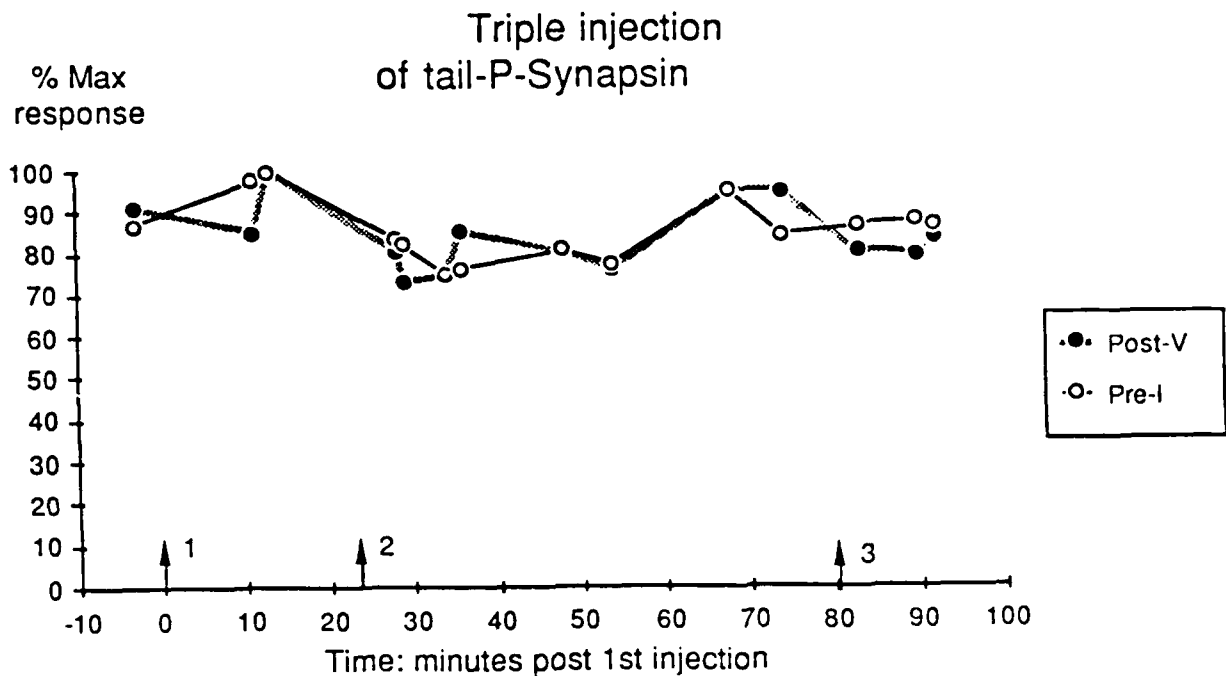
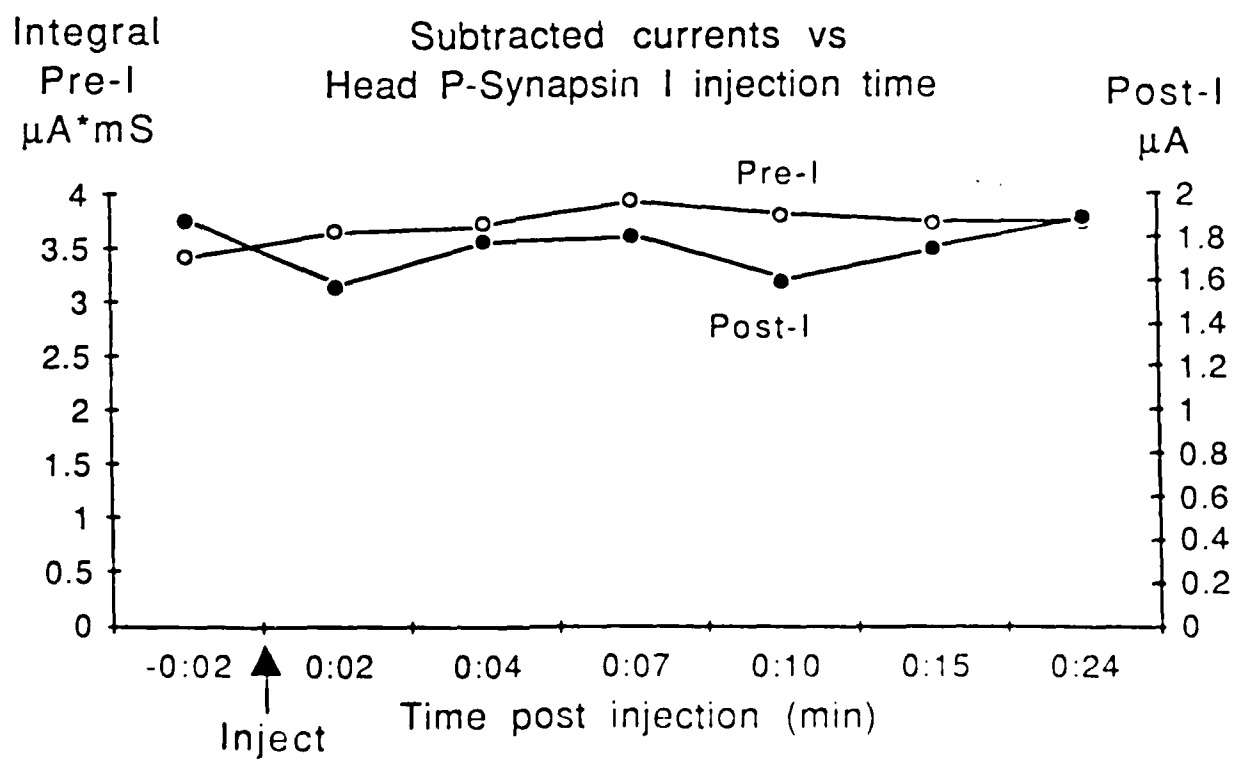
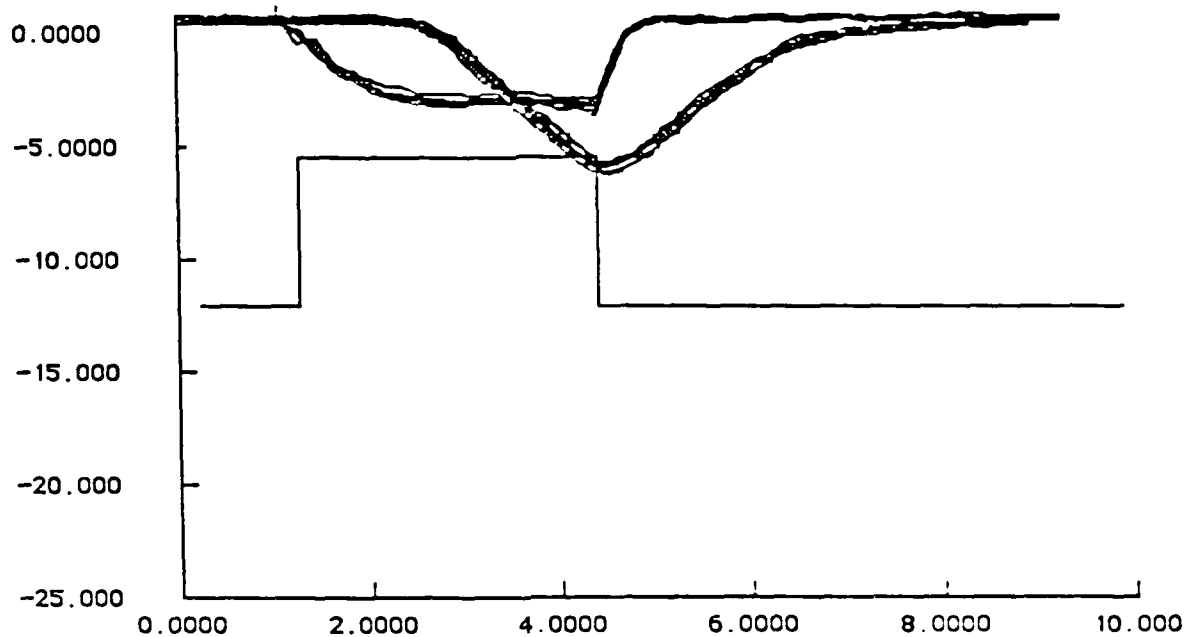


Fig. 1. Presynaptic currents (open circles) and postsynaptic potentials (closed circles) produced by a repeated presynaptic voltage clamp step of constant amplitude following injection of dephospho-tail synapsin I fragment. As shown by the plot the injections which, were monitored by fluorescent microscopy, produced no detectable change in either Ca current or postsynaptic release. The three injections were given at the arrows at times of 0, 23 and 80 minutes. All three injections were large enough to fill all of the terminal. These results indicate that *tail fragment of synapsin I does not produce inhibition of transmitter release.*

Fig. 2. In the upper panel pre- and postsynaptic currents produced by a step voltage depolarization to -35 mV from a holding potential of -65 mV. Results show five sets of voltage clamp recordings indicating no variation in either Ca current or the postsynaptic current at various intervals after the injection of head-phospho synapsin I. At the bottom, plots from a similar set of results taken at different times for 24 minutes following the injection. Note that neither the Ca current nor the postsynaptic current were modified by this injection.



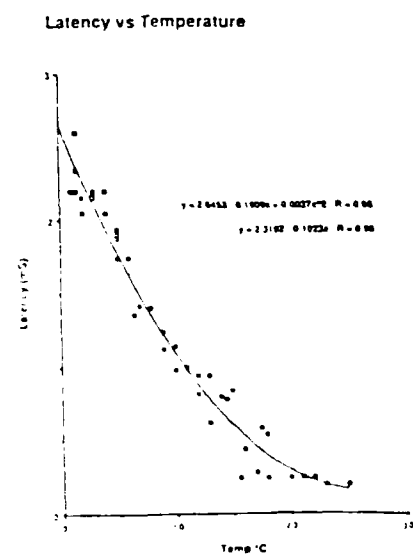
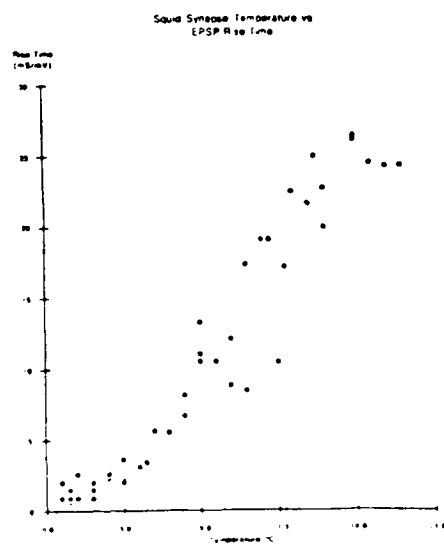
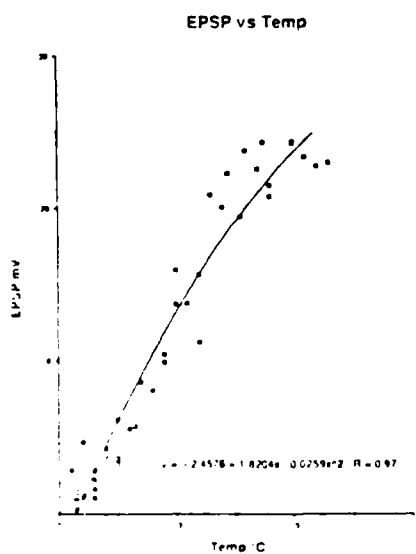
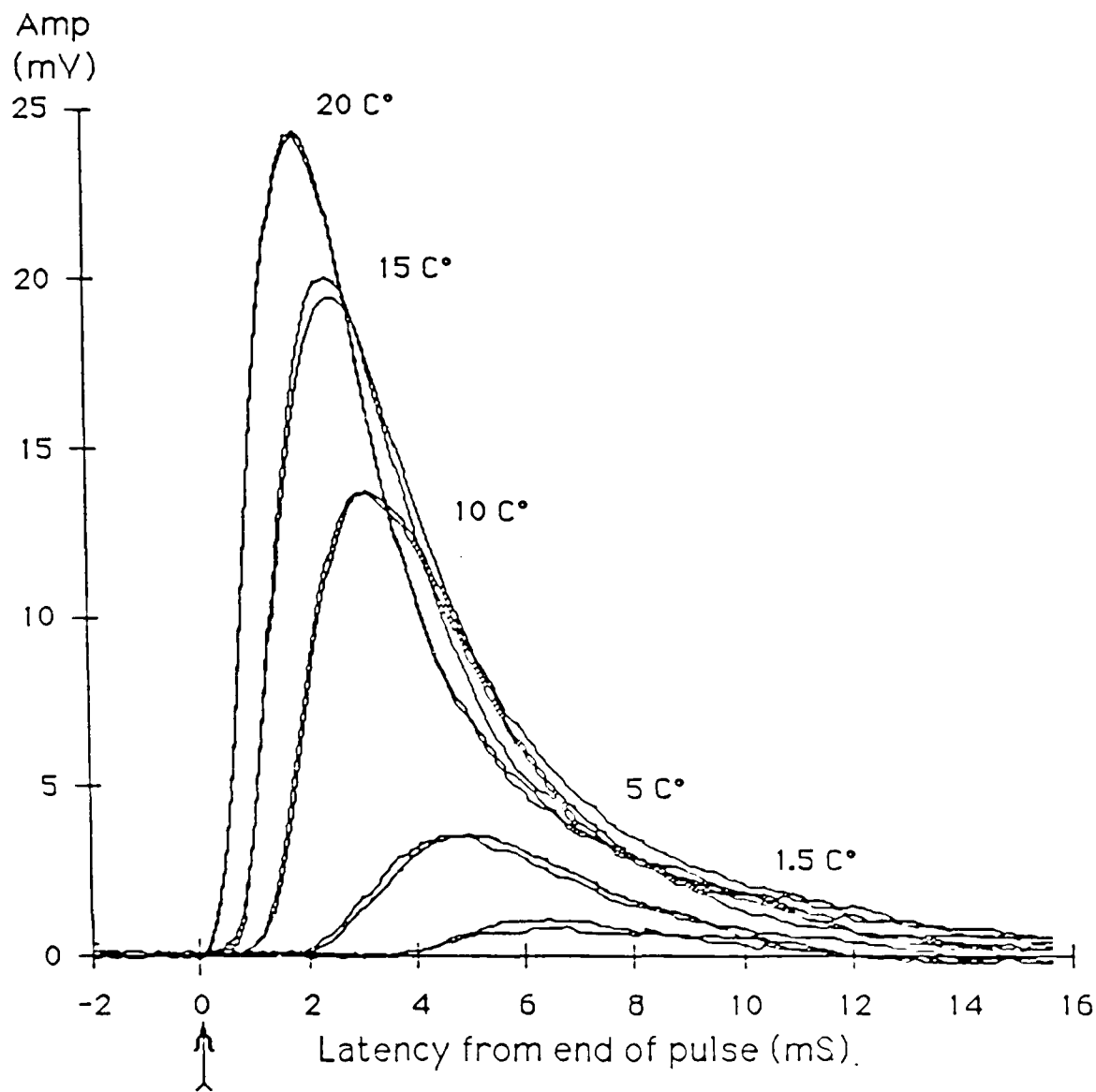
that even in the presence of a complete synapsin I molecule vesicles can fuse. Expts 1 and 2 together demonstrate that the first hypothesis (which is that synapsin I could on its own prevent transmitter release) can be discarded and therefore that the second alternative (that synapsin I regulates transmitter by tethering vesicles to the endoskeletal system) is the most likely explanation for inhibition of transmitter release by synapsin I.

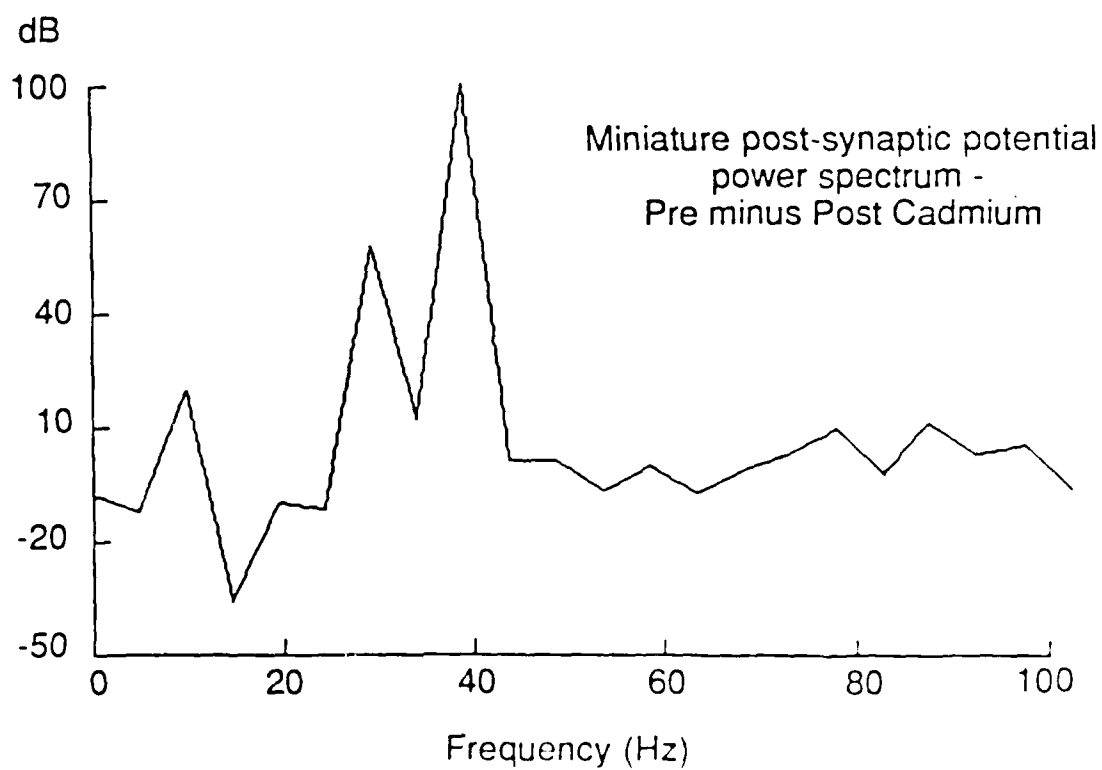
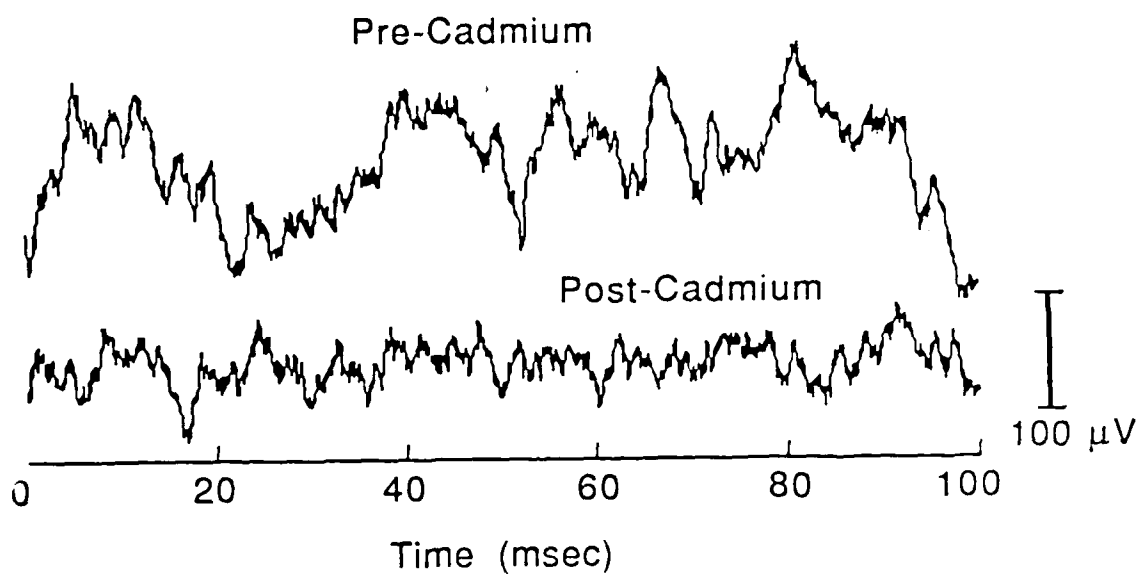
Experiment 3. Having determined the most likely mechanism of synapsin I regulation of synaptic transmission and having confirmed it by video-enhanced microscopy, the next step was to study the molecular mechanisms by which synapsin I tethers synaptic release. Two sets of important measurements must be done in this study in order to proceed with this research. First the temperature dependence on synaptic release in the synapse must be studied in detail as this can give a direct estimate of the equilibrium reactions which regulate the availability of transmitter. The experiments were done following a similar protocol as done for the synapsin experiment. The temperature of the bath was regulated with a Peltier effect system and temperature was modified from 23° to 1° C. The experimental results obtained in one such experiments are illustrated in Fig. 3. Fifty-five measurements were performed in this synapse over a period of 30 minutes at points of temperature shown in the figure. The results indicate that temperature has a well defined effect on synaptic transmission and that latency as long as 2.5 msec after a tail current may be observed in this preparation. This particular set of measurements are of interest on their own but provide an important preliminary measurement to compare degree of blockage and the effect of dephosphorylated synapsin I and the effect that this protein may have on synaptic latency. The results will be utilized in next year's research to determine the temperature dependence of synapsin I on regulation of transmitter release.

Experiment 4. The fourth experiment consisted of direct measurement of the postsynaptic axon utilizing a high gain amplifier demonstrating low noise. The results yielded clearly observable miniature potentials which could be modulated by synaptic depolarization. Several techniques were utilized to obtain these measurements in the large axon as well. The most successful appears to be direct injection of oil in the postsynaptic axon at sufficiently increased input resistance to allow direct observation of these miniature potentials in a large axon.

Fig. 3. Upper panel, postsynaptic potentials generated by a tail Ca current following a voltage clamp step to the suppression potential. At 20° C the postsynaptic potential is very large and has a short latency. Two records are superimposed at each temperature reading. These were taken at different times during the experiment. The results indicate that the amplitude, the latency, and the rate of rise of the postsynaptic potentials are changed by temperature. The plots at the bottom to the left indicate the relation between amplitude of the EPSP and temperature in degrees centigrade, the second the rate of rise against temperature, and the third the latency of onset of the postsynaptic potential against temperature. These three sets of measurements indicate a continuous relation between temperature and the particular parameters plotted. The fit to a second order polynomial is given for the first and last plots.

Fig. 4. High gain recording from postsynaptic axon demonstrating the presence of transient voltage events. The second trace is taken after the addition of CdCl to the bath. The recordings indicate the difference between a synapse that is capable of transmitting and one whose Ca conductance has been blocked. Differences between postsynaptic transients in the presence and absence of Cd were analyzed by fast Fourier transform and indicate that it will be possible to study miniature potentials in this type of preparation. The lower panel indicates power spectrum when the postsynaptic pre-Cd records are subtracted from the post Cd ones.





Status of research

As the 1987 summer research session came to an end some important and clear conclusions were made. We have demonstrated that synapsin I does not act alone, but rather, in conjunction with the endoskeletal system. The molecule seems to be part of a macromolecular chain involving the vesicles and probably at actin filaments. The synapsin I molecule is then the device which keeps vesicle structures near the release site but at a safe distance from the plasmalemmal membrane.

List of accomplishments

(1) Demonstration that the dephosphorylated tail fragments of synapsin I do not on their own regulate synaptic release which excludes the possibility that tail fragments themselves can prevent vesicular release.

(2) Injection of synapsin I phosphorylated at the head does not interfere with synaptic release, demonstrating that even the presence of a whole molecule in contact with the vesicle does not interfere with vesicular fusion. These two experiments taken together indicate that synapsin I regulates transmitter release by their adhesion to endoskeletal systems and that the caging process initially proposed for the regulation of synaptic release is the most likely mechanism for synaptic release regulation.

(3) A set of video-enhanced microscopy results measuring the mobility of axoplasmic organelles demonstrated that both the tail segment of synapsin I added in its dephosphorylated form as well as synapsin I phosphorylated at the head does not interfere with axoplasmic flow, confirming the findings obtained by the above accomplishments (1 and 2). Taken together the results are demonstrative of the actual mechanism for regulation of synapsin I on transmitter release.

(4) Study of the temperature dependence of transmitter release indicates that temperature can be an excellent probe to study the kinetics of synapsin I blockage of transmitter release.

(5) Measurement of miniature potentials may now be utilized as a technique to determine directly the effect of synapsin I on single vesicular fusion.

List of publications

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P. Greengard, M. D. Browning, T. L. McGuinness, and R. Llinas. Synapsin I, a phosphoprotein associated with synaptic vesicles: Possible role in regulation of neurotransmitter release. In Molecular Mechanisms of Neuronal Responsiveness, edited by Y. H. Ehrlich, R. H. Lenox, E. Kornecki and W. O. Berry. New York and London: Plenum Press, 1987.

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T. L. McGuinness, S. T. Brady, R. Llinas, J. Gruner, M. Sugimori and P. Greengard. Phosphorylation-dependent inhibition by synapsin I of membranous organelle movement in extruded squid axoplasm. Submitted for publication.

List of professional personnel

Rodolfo R. Llinas, Principal Investigator
M. Sugimori, Investigator
John Gruner, Investigator

Supporting staff: Alfred Benedek, Electronics Design Specialist
Gregory Mahoney, Programming Specialist

Collaborators: Theresa L. McGuinness (Rockefeller University)
Paul Greengard (Rockefeller University)
Scott Brady (University of Texas, Dallas)

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